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## Structural Insight into Antibiotic Fosfomycin Biosynthesis by a Mononuclear Iron Enzyme

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*The mononuclear iron enzyme S-(2)-hydroxypropylphosphonic acid epoxidase (HppE), from Streptomyces wedmorensis, uses $O_2$ to catalyze the formation of the broad-spectrum antibiotic fosfomycin (which inhibits bacterial cell-wall peptidoglycan biosynthesis) from S-(2)-hydroxypropylphosphonic acid (S-HPP). The reaction is a two-electron oxidation and is mechanistically atypical because it is independent of any cofactor or co-substrate and results in the incorporation of the hydroxyl oxygen of the substrate, rather than an atom of $O_2$, into the epoxide ring. The x-ray crystal structures of six forms of HppE — apo-HppE, Fe(II)-HppE, tris(hydroxymethyl)aminomethane-Co(II)-HppE complex, S-HPP-Co(II)-HppE complex, and two S-HPP-Fe(II)-HppE complexes — were solved using data collected in part at the NSLS. The purpose of this was to gain insight into the mechanism of this unique enzyme.*

Mononuclear non-heme iron enzymes use their metal cofactor to activate dioxygen ($O_2$) for difficult redox processes. One of these enzymes, *S*-(2)-Hydroxypropylphosphonic acid epoxidase (HppE), from *Streptomyces wedmorensis* (**Figure 1**, overall structure), employs its mononuclear iron center and molecular oxygen for the two-electron oxidation of *S*-(2)-hydroxypropylphosphonic acid (*S*-HPP) to catalyze the formation of the antibiotic (1R,2S)-(1,2-epoxypropyl)phosphonic acid (fosfomycin). This reaction is essentially a dehydrogenation reaction (loss of hydrogen). In order to balance the four-electron reduction of oxygen to water, we have proposed a putative two-electron reductant (or reductase). Fosfomycin is an unusual C-P-bond-containing epoxide that covalently modifies UDP-GlcNAc enolpyruvyl transferase, consequently inhibiting bacterial cell-wall peptidoglycan biosynthesis and bacterial growth. Since it accumulates in the kidneys and bladder, fosfomycin has been used clinically for the treatment of lower-urinary-tract infections. The structures of the apo-HppE (metal-free), native Fe(II)-HppE, tris(hydroxymethyl)aminomethane (Tris)-Co(II)-HppE complex, *S*-HPP-Co(II)-HppE complex, and two *S*-HPP-Fe(II)-HppE complexes (form 1 and form 2) were solved in order to better understand the epoxidation mechanism of this enzyme. It is interesting to note that the Tris molecules in the Tris-Co(II)-HppE structure result from the Tris buffer used in the crystallization solution.

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Selenomethionine (SeMet) derivatization was used to obtain phase information for these protein structures. Initial experimental phases were determined from an x-ray dataset of Tris-Co(II)-SeMet-HppE collected at the wavelength for the Se absorption peak (0.9791 Å) on NSLS beamline X26C. This dataset was refined to 2.5 Å in space group $P6_522$ and the resulting model was further refined against a 1.8 Å native Tris-Co(II)-HppE dataset that was obtained at 0.9791 Å on Advanced Photon Source beamline 8BM. All subsequent structures were determined from these initial models.

The structures of *S*-HPP-Fe(II)-HppE complexes (form 1 and form 2) confirm the direct binding of the substrate, *S*-HPP, to the iron and show the existence of two binding modes, a monodentate mode and a bidentate mode. These two modes are explained by a two-step binding process: (i) *S*-HPP first binds in a monodentate fashion via the oxygen atom of the phosphonic acid group, resulting in displacement of a water molecule; (ii) The subsequent rotation of the substrate allows for bidentate coordination of *S*-HPP to Fe(II). A

β-hairpin-like structure, formed by β-strands 2 and 3, acts as a cantilever that responds to the bidentate positioning of the substrate and adopts a closed catalytic conformation to cover the hydrophobic portion of the substrate bound in the active site (**Figure 2**). The negative charge on the substrate is expected to enhance the reactivity of the diiron center toward oxygen, a role that co-substrates play in other mononuclear iron proteins. The addition of oxygen to the only open coordination site on Fe(II) of the bidentate-S-HPP complex appears to occur through a very small channel created at the interface of the α- and β- domains. Once $O_2$ is bound, abstraction of the C1 hydrogen atom could occur by a Fe(IV)-oxo intermediate (**Scheme 1, pathway A**) or by a Fe(III)-hydroperoxide intermediate (**Scheme 1, pathway B**). This results in the formation of a transient-substrate radical intermediate that undergoes cyclization to yield fosfomycin.

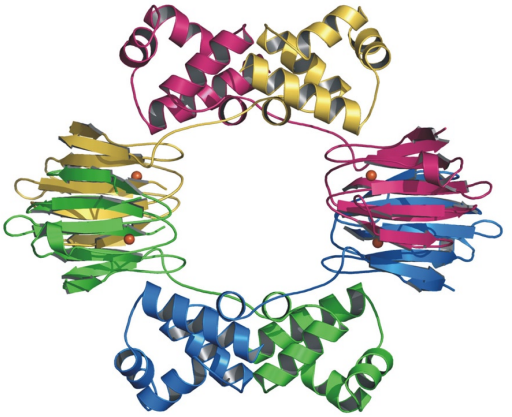


***Figure 1.*** *Tetrameric structure of HppE.*

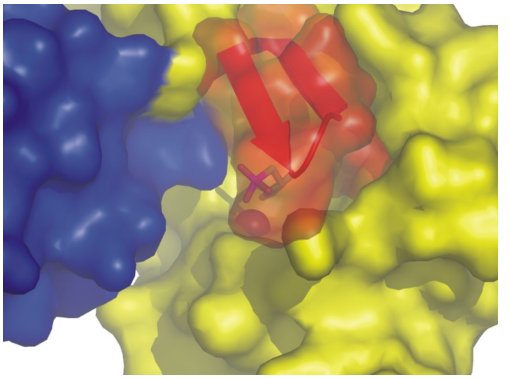
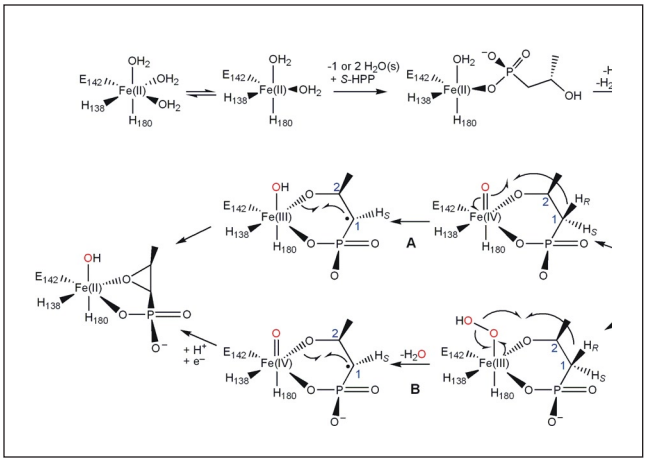


***Figure 2.*** *A portion of the structure called the cantilever (red) closes over the active site iron (brown) and substrate (ball-and-stick).*




***Scheme 1.*** *Possible reaction mechanisms for HppE.*